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In Vitro Genotoxicity Test of *m*-Phenylenediamine Using the Chromosomal Aberration Assay in Cultured Mammalian Cells

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Abstract

On February 21, 2023, the Ministry of Food and Drug Safety in Korea designated five hair dye ingredients, including m-phenylenediamine, as prohibited substances in cosmetics due to potential genotoxicity, which may cause genetic damage or mutations in humans. Based on the revised regulations, products containing these ingredients have been banned from manufacture and import as of August 22, 2023. However, products manufactured or imported before this date may still be sold until August 21, 2025. Therefore, m-phenylenediamine, which has already been verified to exhibit genotoxicity, was selected as the test substance

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for this study.

The chromosomal aberration test was conducted in compliance with OECD TG 473. Mammalian cell lines, specifically the Chinese Hamster Lung (CHL/IU) cell line, were used. The test examined the chromosomal aberration-inducing potential of the test substance with and without metabolic activation. Genotoxicity assessment is not conducted based on a single test; instead, it requires a 3-battery approach, comprising from three different tests. Preliminary studies have shown a positive response in the reverse mutation assay for m-phenylenediamine. This study adds the chromosomal aberration test to compare the results, supporting the validity of a 3-battery approach and suggesting the ethical viability of cell-based toxicity testing to assess chemical hazards.

Keywords: *m-Phenylenediamine, Genotoxicity, Chromosomal Aberration Assay, In Vitro Toxicology, Mammalian Cell Culture*

1. Introduction

m-Phenylenediamine, also known as 1,3-diaminobenzene, MPD, or MPDA, is an organic compound. It is an isomer of o-phenylenediamine and p-phenylenediamine, and typically appears as a colorless solid. However, upon exposure to air, it undergoes oxidation, which causes it to turn red or purple[1]. *m*-Phenylenediamine is produced by hydrogenating 1,3-dinitrobenzene, which itself is synthesized by the dinitration of benzene[2]. This compound is used in the production of various polymers, including aramid fibers, epoxy resins, wire enamel coatings, and polyurea elastomers. Other uses include its role as an accelerator in adhesive resins and as a component in dyes for leather and textiles. *m*-Phenylenediamine is also used as a binding agent to create a blue color in human hair dyes.

A chromosomal aberration test using cultured mammalian cells was conducted at a domestic good laboratory practices (GLP) facility in accordance with OECD TG 473[3]. The chromosomal aberration-inducing potential of the test substance was evaluated using a cultured mammalian cell line, Chinese Hamster Lung (CHL/IU) cells, under both metabolic activation and non-activation conditions. CHL/IU cells are widely used in genotoxicity tests and abundant background data is available, facilitating data interpretation and accessibility [4]. In addition, these cells are recommended in the OECD Guidelines for Testing of Chemicals TG 473[5].

For cell preservation, the harvested cells were inoculated into culture media and incubated in a 5% CO2 incubator at 37 ° C. After incubation, the cells were separated from the culture vessel by adding 0.05% trypsin-ethylenediaminetetraacetic acid (EDTA) and then centrifuged at 1,000 rpm for 5 min. The supernatant was removed, and the cells were suspended in Eagle's Minimum Essential Medium (MEM) containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (P/S), and glutamine (Glu). The cell suspension was mixed with FBS and dimethyl sulfoxide (DMSO), dispensed into cryogenic tubes for preservation, frozen at ultra-low temperatures (−80 to −60 ° C) for over 4 h, and then stored in a nitrogen tank.

Mitomycin C (MMC) was used as the positive control for the non-metabolic activation test, whereas 3,4-Benzpyrene (B[a]P) was used for the metabolic activation test. Sterilized distilled water for injection served as the negative control and vehicle[6].

2. Experiments

The preparation of positive control substances for each strain was based on historical control data. B[a]P

and MMC were prepared in sterile distilled water for injection, aliquoted into storage tubes, and stored in an ultralow-temperature freezer. On the treatment day, the required amount was thawed for use[7].

Table 1. Preparation of Positive Control Substances

Treatment	S9 mix	Control	Concentration (μg/mL)
Short-term	-	MMC	0.2
	+	B[a]P	20
Continuous	-	MMC	0.2

The metabolic activation system (S9 mix) was prepared as follows: In the dose-setting test, 1.0 mL of S9, 2.5 mL of cofactor, and 1.5 mL of sterile distilled water (Gibco, U.S.A) were mixed to prepare 5.0 mL of 20% S9 mix. For the main test, 1.2 mL of S9, 3.0 mL of cofactor, and 1.8 mL of sterile distilled water were mixed to prepare 6.0 mL of 5% S9 mix[8].

Table 2. Preparation of Metabolic Activation System

Component	Substance	Concentration
S9 (20% v/v)	S9 fraction	0.2 mL/mL
Cofactor (80% v/v)	$MgCl_2$	8 μmol/mL
	KCl	3 μmol/mL
	G-6-P	5 μmol/mL
	Sodium phosphate buffer (pH 7.4)	5 μmol/mL

A dose-setting test was conducted to determine the optimal dose for the main test. One 60-mm plate (4 mL/plate) was used per dose, and each plate was labeled with the dose and whether the S9 mix was present. After counting the subcultured cells, they were diluted to a concentration of 1×10^4 cells/mL, dispensed onto each plate, and pre-incubation in a 5% CO_2 incubator at 37 °C for 3 days. AIn addition, an untreated satellite group was cultured, and the cell count was measured before treatment with the test substance[9].

Table 3. Main Test Dosage Based on Dose-Setting Test Results

Treatment	S9 mix	Concentration (μg/mL)
Short-term	-	610, 305, 153, 76.3
	+	1,000, 500, 250, 125
Continuous	-	95.0, 47.5, 23.8, 11.9

The main test was conducted using the same methods and conditions as those used for the dose-setting test. However, in the main test, two plates were used for each dose.

Table 4. Preparation Volume and Dispensing Volume for Each Treatment Group

Treatment	S9 mix	Group	Preparation Volume (mL)			Concentration (μg/mL)
			Media	S9 mix	Substance	
Short-term	-	NC	8.10	-	0.90	4
		TC	8.10		0.90	4
		PC	8.91		0.09	4
	+	NC	7.20	0.9	0.90	4
		TC	7.20		0.90	4
		PC	8.01		0.09	4
Continuous	-	NC	8.10	-	0.90	4
		TC	8.10		0.90	4
		PC	8.91		0.09	4

The test substance treatment was conducted following the same process as the dose-setting test, with no observed changes in osmotic pressure or pH. Colcemid solution (0.2 μg/mL) was added 2 h before the end of the incubation. After incubation, the cells were washed with phosphate-buffered saline (PBS), treated with 0.05% trypsin-EDTA solution, and detached from the plate. The cells were then centrifuged at 1,000 rpm for 5 min and the supernatant was discarded. Next, 3 mL of 0.075 mol/L KCl solution, pre-warmed to 37 °C, was added, and the mixture was treated at 37 °C for 30 min[10]. One milliliter of chilled fixative (methanol:acetic acid, 3:1) was added, and the cells were centrifuged at 1,000 rpm for 5 min to remove the supernatant, achieving partial fixation. Subsequently, 5 mL of chilled fixative was added, and the cells were centrifuged at 2,000 rpm for 5 min. This process was repeated once more to complete fixation. The cell suspension was then dropped onto glass slides to prepare specimen slides, with two slides per plate. The slides were then completely dried and stained with 5% Giemsa stain for 20 min. After staining, the slides were rinsed with distilled water and dried[11].

3. Results and Discussion

The chromosomal aberration test was considered valid as it met the following criteria: In the negative control group, the frequency of cells with chromosomal structural aberrations fell within the 95% control range of historical control data. In the positive control group, the frequency of cells with chromosomal structural aberrations was within the historical control data range and showed a statistically significant increase compared with that in the negative control group. At least three analyzable doses were tested, and no contamination occurred[12].

Table 5. Summary Results of the Dose Range Finding Study(RPD)

Test substance	S9 mix /Treatment	Dose (μg/mL)	Relative Population Doubling (%)
NC	S9+ /6hr	0	100.0
TC		62.5	89.6
		125	92.3
		250	63.2
		500	59.9

		1,000	14.1
		2,000	-45.9
NC	S9-	0	100.0
TC	/6hr	62.5	88.7
		125	101.8
		250	90.7
		500	88.1
		1,000	76.3
		2,000	-22.3
NC	S9+	0	100.0
TC	/24hr	62.5	38.8
		125	42.1
		250	8.7
		500	20.8
		1,000	8.7
		2,000	-61.6

Table 6. Summary Results of the Main Study(Numerical Aberrations)

Test substance	S9 mix /Treatment	Dose (μg/mL)	Number of cells with numerical aberrations
NC	S9+	0	0
TC	/6hr	76.3	0
		153	0
		305	0
		610	0
PC		20	0
NC	S9-	0	0
TC	/6hr	125	0
		250	0
		500	0
		1,000	0
PC		0.2	0
NC	S9+	0	0
TC	/24hr	11.9	0
		23.8	0
		47.5	0
		95.0	0
PC		0.2	0

In both the short-term treatment with and without metabolic activation and the continuous treatment without metabolic activation, no increase in the frequency of chromosomal aberrant cells induced by the test substance were observed compared to that in the negative control across all dose levels. In contrast, the positive group for each treatment series showed a statistically significant increase in the frequency of chromosomal aberrant cells compared with that in the negative control, indicating a clear positive result[13].

4. Conclusion

In this study, to demonstrate the validity of the genotoxicity 3-battery test, we conducted a chromosomal aberration test and a micronucleus test on *m*-Phenylenediamine, a substance that tested positive in the reverse mutation test[14].

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While the reverse mutation test showed a positive result, both the chromosomal aberration test and the micronucleus test showed negative results. Conducting only a single genotoxicity test may lead to erroneous results, as seen in this study[15]. Moreover, the presence or absence of toxicity could be determined through cell-based assays rather than animal experiments, which involve ethical concerns.

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